

Continuous butanol fermentation and feed starch retrogradation: butanol fermentation sustainability using *Clostridium beijerinckii* BA101

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Abstract

Use of starch solution as feed for butanol bioconversion processes employing *Clostridium beijerinckii* BA101 may have added economic advantage over the use of glucose. Acetone butanol ethanol (ABE) was produced from 30 g L⁻¹ starch solution using a continuous process. The bioreactor was fed at a dilution rate of 0.02 h⁻¹ and starch solution/feed volume (3 L) was replaced every 72 h. The continuous reactor fed with cornstarch solution (feed temperature 19 °C) produced approximately 6.0 g L⁻¹ total ABE. Increasing the feed storage temperature to 37 °C improved ABE production to 7.2 g L⁻¹ suggesting that retrogradation was occurring more rapidly at 19 °C. In both these cases the fermentation drifted toward acid production after approximately 260 h, consistent with the retrogradation of starch overtime. The use of soluble starch, which is less prone to retrogradation, resulted in the production of 9.9 g L⁻¹ ABE at 37 °C feed storage temperature, as compared to 7.2 g L⁻¹ ABE when cornstarch was used. It should be noted that gelatinized starch retrogradation takes place after sterilization and prior to use of the feed medium, and does not occur during long-term storage of the raw corn material in the months leading up to processing. The degree of hydrolysis of gelatinized starch decreased from 68.8 to 56.2% in 3 days when stored at 37 °C. Soluble starch which does not retrograde demonstrated no change in the degree of hydrolysis.

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1. Introduction

The acetone butanol ethanol (ABE) fermentation, which was an important industrial process during the

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first half of 20th century, was based on the use of starchy substrates such as corn. Butanol, which is an excellent biofuel, has numerous other applications in the food, plastics, and chemical industries. In a recent economic study, we discussed the role of fermentation substrate on the price of butanol (Qureshi and Blaschek, 2000, 2001). Starch and starch-based co-products can be used for conversion to fuels and chemicals such as butanol. The production of amylolytic enzymes (α -amylases and amyloglucosidases) by the production microorganism, known as simultaneous saccharification and fermentation, has an advantage over systems where hydrolysis and subsequent fermentation of starch occur separately (Jesse et al., 2002). In the early 1990s, our laboratory developed a hyper-amylolytic, hyper-butanol producing solventogenic *Clostridium* designated *C. beijerinckii* BA101 that is efficient in conversion of starch to acetone-butanol (Annous and Blaschek, 1991). The genetic amplification of the extracellular amylases allows for simultaneous saccharification and fermentation of starch-based biomass to butanol (Formanek et al., 1997).

Although a significant number of studies have been carried out on ABE production when using starch as carbon source, most if not all, have employed batch fermentation processes (Madiah et al., 2001; Soni et al., 1992). Since utilization of starch for solvent production involves a two-step process: [(a) breakdown of starch by α -amylase (Sigma, St. Louis, MO) to oligosaccharides and hydrolysis of oligosaccharides by de-branching enzymes such as glucoamylase or amyloglucosidases to glucose, and (b) subsequent fermentation of glucose to solvent], long-term continuous cultivation of a solventogenic culture in a starch-based medium may not be an easy task. Starch granules are insoluble in water and resistant to enzymatic hydrolysis. Starch gelatinizes when heated in the presence of excess water. Gelatinization involves the collapse of starch granules, which is manifested as irreversible changes in properties such as native crystallite melting, loss of birefringence and starch solubilization (Atwell et al., 1988; Nwokocha, 2002). Gelatinized starch gels are thermo-dynamically unstable structures, and on cooling re-association of the starch molecules through H-bonding involving both amylose and amylopectin occurs, with a corresponding increase in viscosity, a phenomenon termed retrogradation. The rate of retrogradation depends on a number of variables includ-

ing the structure of amylose and amylopectin, the ratio of amylose to amylopectin, temperature, concentration of starch, botanical source of starch, and presence and concentration of other ingredients (Kim et al., 1997; Jacobson et al., 1997). Retrogradation has been shown to significantly lower the enzymatic susceptibility of gelatinized starch to hydrolysis (Eerlingen et al., 1994; Cui and Oates, 1997; Garcia-Alonso et al., 1999; Fredriksson et al., 2000). Starch modification is often carried out to alter the physical and chemical properties of the native starch in order to decrease retrogradation and improve the functional characteristics of starch paste (BeMiller, 1997; Nwokocha, 2002).

Since starch retrogradation occurs upon cooling of autoclaved starch solution, in particular when feed solution is stored for extended periods of time, as would be the case with continuous cultures, the objective of this study was to investigate the long-term continuous cultivation of solvent producing hyper-amylolytic *C. beijerinckii* BA101 in starch-based medium for butanol production. An additional objective was to examine the effect of starch retrogradation on sustainability of the ABE fermentation.

2. Materials and methods

2.1. Bacterial strains and culture maintenance

C. beijerinckii BA101 was used for these studies (Annous and Blaschek, 1991). Laboratory stocks of *C. beijerinckii* BA101 were routinely maintained as spore suspensions in sterile ddH₂O at 4 °C. Spores (200 μ L) were heat shocked for 10 min at 80 °C followed by cooling in the inoculation chamber for 5 min. The culture was inoculated into 25 mL cooked meat medium (CMM) contained in 50 mL screw capped Pyrex bottle and was incubated anaerobically for 16 h at 36 ± 1 °C. Six milliliters of culture was transferred into 100 mL tryptone-glucose-yeast extract (TGY) medium and incubated for 4–6 h until an optical density at 600 nm (Beckman Du 640 spectrophotometer) of 1.2–1.6 was achieved.

2.2. Preparation of medium

To prepare the medium for continuous fermentation, 30 g L⁻¹ of corn or soluble starch and 1 g L⁻¹

yeast extract (Difco) were dissolved in distilled water followed by heating and stirring in water bath at 100 °C until boiling. This was followed by sterilizing at 121 °C for 15 min. The medium was cooled by flushing O₂-free N₂ gas across the surface. On cooling to 37 °C, filter-sterilized P2 medium stock solutions (Qureshi and Blaschek, 1999) were added (16 mL of each of these solutions to 1472 mL of autoclaved medium containing starch and yeast extract): (1) buffer: K₂HPO₄, 50 g L⁻¹; ammonium acetate, 220 g L⁻¹. (2) Vitamins: Para-amino-benzoic acid, 0.1 g L⁻¹; thiamin, 0.1 g L⁻¹; biotin, 0.001 g L⁻¹. (3) Mineral: MgSO₄·7H₂O, 20 g L⁻¹; MnSO₄·H₂O, 1 g L⁻¹; FeSO₄·7H₂O, 1 g L⁻¹; NaCl, 1 g L⁻¹. The stock solutions were filter sterilized through a 0.2 µm pore-size polyethersulfone membrane filter unit (Nalgene, Rochester, NY). The feed (fermentation medium) was maintained in an airtight bottle provided with an inlet and outlet for O₂-free N₂ gas to maintain anaerobiosis. N₂ was flushed across the surface of the medium continuously. The feed was kept at either room temperature (19 °C) or 37 °C and stirred at 100 rpm to avoid sedimentation.

2.3. Batch fermentations

Batch fermentations were carried out in 150-mL screw-capped bottles (125 mL of medium). The medium contained corn or soluble starch at 40 g L⁻¹, yeast extract at 1 g L⁻¹, and was autoclaved in bottles. On cooling, the medium was transferred to an anaerobic chamber (Coy, Ann Arbor, MI) at 36 °C for 24 h for anaerobiosis. This was followed by the addition of P2 stock solution and inoculation with a 5% (v/v) actively growing TGY cell suspension. Fermentation proceeded at 36 ± 1 °C until complete. During the course of fermentation, 1-mL samples were collected regularly for ABE, acids, and starch analysis.

2.4. Continuous cultivation of *C. beijerinckii* BA101

Continuous cultivation of *C. beijerinckii* BA101 was carried out in P2 medium (Qureshi and Blaschek, 1999) containing 30 g L⁻¹ soluble starch or cornstarch in a New Brunswick 2-L continuous culture bioreactor (New Brunswick Scientific Co., New Brunswick, NJ) incubated at 36 °C. The bioreactor was inoculated

with a 5% (v/v) inoculum of highly motile cells of *C. beijerinckii* BA101. The fermentation was allowed to proceed in the batch mode for 24 h, after which P2 medium containing either corn or soluble starch were continuously pumped into the bioreactor at a dilution rate of 0.019 h⁻¹. The bioreactor was maintained at 36 ± 1 °C. The temperature of the feed was maintained at either room temperature (19 °C) or 37 °C. Fresh feed was prepared every 3 days. The fermentation could not be run with 40.8 g L⁻¹ starch feed solutions due to the viscous nature of the feed. However, batch fermentation could be carried out when using 40 g L⁻¹ starch solution concentration. In continuous cultures, the utilization of 30 g L⁻¹ feed starch was incomplete. Based on the evidence that neither of the fermentations (batch or continuous) were substrate limited and not toxic to the culture at starch feed levels of 30 and 40 g L⁻¹, these comparisons should be valid.

2.5. Enzymatic hydrolysis of starch

A mixture of 1.0 mL of 2.0% (w/v) gelatinized starch and 1.0 mL of 0.1 M phosphate buffer (pH 5.5) were equilibrated at 50 °C in a water bath for 5 min. 50 µL α-amylase solution was added and the sample was further incubated for 45 min and boiled for 5 min to stop the reaction. Reducing sugar was determined using the 3,5-dinitrosalicylic acid (DNSA) method (Bergmeyer and Grassel, 1983).

2.6. Acid hydrolysis of starch

Hydrolysis was carried out by incubating 2.0% starch with 1 M HCl at 100 °C for 2 h. The sample was assayed immediately for reducing sugars. The degree of hydrolysis (DH) (Cui and Oates, 1997) was calculated as follows:

$$\text{DH}(\%) = \frac{\text{reducing sugar produced by amylase hydrolysis}}{\text{reducing sugar produced by acid hydrolysis}} \times 100$$

2.7. Analytical procedures

ABE and acids (acetic and butyric) were measured using a 6890 Hewlett-Packard gas chromatograph

(Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector (FID) and 6 ft \times 2 mm glass column (10% CW-20M, 0.01% H₃PO₄, support 80/100 Chromosorb WAW). Productivity was calculated as total ABE (solvent) concentration (g L⁻¹) multiplied by the dilution rate (mL h⁻¹).

Glucose concentration was determined using a hexokinase and glucose-6-phosphate dehydrogenase (Sigma) coupled enzymatic assay. The fermentation medium was centrifuged (microfuge centrifuge) at 10,000 \times g for 3 min at 4 °C. A portion of the supernatant (10 μ L) was mixed with glucose (HK) 20 reagent (1.0 mL) and incubated at room temperature for 5 min. Standard solutions of anhydrous D-glucose containing 1–5 g of glucose L⁻¹ of distilled water were prepared. Ten microliters of each of the standard solution was mixed with glucose (HK) 20 reagent (1.0 mL) and incubated at room temperature for 5 min. A blank (deionized water) (10 μ L) was incubated with the reagent and was used for zero adjustment of the spectrophotometer. After 5 min, the absorbance was measured at 340 nm using a Beckman DU 640 spectrophotometer and the glucose content in the sample was computed by least squares linear regression using a standard curve.

Starch concentrations of the samples were determined using a modified method of Holm et al. (1986). The sample (250 mg) was suspended in distilled water (15 mL) in a 50-mL beaker. Heat-stable α -amylase (100 μ L) (Sigma) was added and mixed gently with a magnetic stirrer. The beaker was placed in a boiling water bath for 30 min with mixing every 5 min. The suspension was allowed to cool under continuous agitation on a magnetic stirrer and was transferred to a 25-mL volumetric flask followed by filling it with distilled water to the volume. One milliliter of the solution was transferred to a test tube followed by adding 2.9 mL of 0.1 M sodium acetate buffer (pH 4.75) and 100 μ L of amyloglucosidase (Sigma). The mixture was incubated for 60 min at 55 °C with mixing every 5 min. The sample was then transferred to a 50-mL volumetric flask and filled to volume with distilled water. Ten microliters of the solution was assayed for glucose according to the hexokinase and glucose-6-phosphate dehydrogenase assay method described above:

$$\text{Starch (\%)} = \frac{\text{mg glucose} \times 25^a \times 50^a \times 0.9^b \times 100}{\text{sample weight (250 mg)}}$$

where a is dilution factor and b is correction glucose to glucan.

3. Results

3.1. Batch fermentation

In order to evaluate the fermentation characteristics of starch solutions, a batch fermentation using *C. beijerinckii* BA101 was run with P2 medium containing 40.8 g L⁻¹ starch. The fermentation was rapid. However, because of the opaque nature of starch, measurement of cell concentration by optical density was not possible. Over the course of 72 h, the culture produced 5.4 g L⁻¹ acetone, 14.3 g L⁻¹ butanol, and 0.3 g L⁻¹ ethanol, resulting in a total ABE concentration of 20.0 g L⁻¹ (Table 1). The starch concentration of the medium decreased from 40.8 to 3.6 g L⁻¹ (Fig. 1). Solvent productivity and residual total acids were 0.28 g L⁻¹ h⁻¹ and 1.65 g L⁻¹, respectively. The batch fermentation data presented herein represents the average of duplicate determinations. These results demonstrate that starch can be successfully utilized by *C. beijerinckii* BA101 for production of ABE.

3.2. Effect of corn and soluble starch on continuous cultivation of *C. beijerinckii* BA101

In order to evaluate the sustainability of the ABE fermentation in a continuous process using corn starch-based P2 medium and *C. beijerinckii* BA101, a continu-

Table 1
Batch fermentation of corn starch to ABE by *C. beijerinckii* BA101

Parameters	Glucose ^a (control)	Corn starch
Acetone (g L ⁻¹)	5.31	5.4
Ethanol (g L ⁻¹)	0.5	0.3
Butanol (g L ⁻¹)	11.9	14.3
Residual acetic acid (g L ⁻¹)	0.5	1.4
Residual butyric acid (g L ⁻¹)	0.4	0.3
Total ABE (g L ⁻¹)	17.7	20.0
Total acids (g L ⁻¹)	0.9	1.7
Initial substrate (g L ⁻¹)	59.2	40.8
Residual substrate (g L ⁻¹)	14.6	3.6
Fermentation time (h)	60	72
Solvent yield (g g ⁻¹)	0.4	0.49
Productivity (g L ⁻¹ h ⁻¹)	0.29	0.28

^a Data obtained from Ezeji et al. (2003).

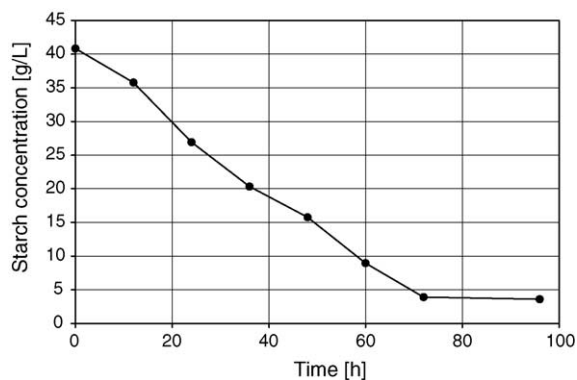


Fig. 1. Corn starch utilization overtime during batch cultivation of *C. beijerinckii* BA101 in semidefined P2 medium containing corn starch.

ous reactor whose feed supply was maintained at room temperature (19 °C) was run at a flow rate of 30 mL h⁻¹ (dilution rate 0.019). The fermentation terminated after 312 h at which time 5.08 g L⁻¹ total acids, were present in the effluent. Results for total solvent production recorded over the course of the fermentation process are shown in Fig. 2a. For the first 240 h, the fermentation was active as indicated by high gas production but then slowed down. Following 240 h of operation, the fermentation started deteriorating, the residual glucose concentration in the bioreactor decreased (Fig. 2b) and there was drift toward acid production. At this stage, it was observed that significant amounts of sediment (retrograded starch) had accumulated in the bottom of the reactor (up to 7.2 cm high; bioreactor vessel height, 21.5 cm). To determine if fermentation had stopped owing to the toxicity of the sediments to the culture, the reactor liquid was drained into a separating funnel and the sediments were separated. The volume of the sediments was made up to reaction volume (1600 mL) with distilled water. To this volume 96 g of glucose (60 g L⁻¹) and 1.6 g of yeast extract (1 g L⁻¹) were added followed by autoclaving. Following cooling overnight in the anaerobic chamber, P2 medium stock solutions were added. The mixture was distributed into 150-mL screw-capped bottles (reaction volume, 105 mL) inoculated with a freshly grown culture. Over the course of 72 h, the culture produced 4.01 g L⁻¹ acetone, 11.98 g L⁻¹ butanol, and 0.31 g L⁻¹ ethanol, resulting in a total ABE concentration of 16.30 g L⁻¹, suggesting that the retrograded

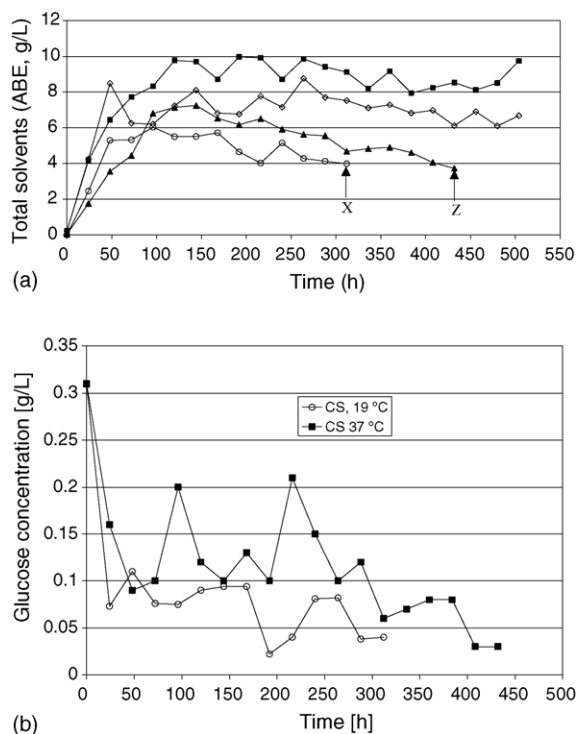


Fig. 2. (a) Solvent concentration values obtained during continuous cultivation of *C. beijerinckii* BA101 in semidefined P2 medium containing corn or soluble starch. Symbols: (○) corn starch (19 °C); (▲) corn starch (37 °C); (◇) soluble starch (19 °C); (■) soluble starch (37 °C). Arrows show the times (points X and Z) when fermentation terminated in the bioreactor. (b) Residual glucose concentrations obtained during continuous cultivation of *C. beijerinckii* BA101 in semidefined P2 medium containing corn starch. Symbols: (○) corn starch (19 °C); (■) corn starch (37 °C).

starch sediments per se are not toxic to *C. beijerinckii* BA101 (Fig. 3). The fermentation of corn starch whose feed supply was maintained at 37 °C terminated at 432 h. In terms of solvent production, the fermentation whose feed supply was maintained at 37 °C was better than the fermentation whose feed supply was maintained at 19 °C (Fig. 2a).

To further investigate why the fermentation of corn starch in P2 medium terminated after 312 h (with feed temperature at 19 °C), two continuous reactors whose feed supply was maintained at 19 and 37 °C were operated with P2 medium containing 3% soluble starch. The continuous process with feed temperature at 37 °C reached a steady state after 120 h resulting in 9.8 g L⁻¹ ABE and 0.2 g L⁻¹ h⁻¹ productivity, with only minor

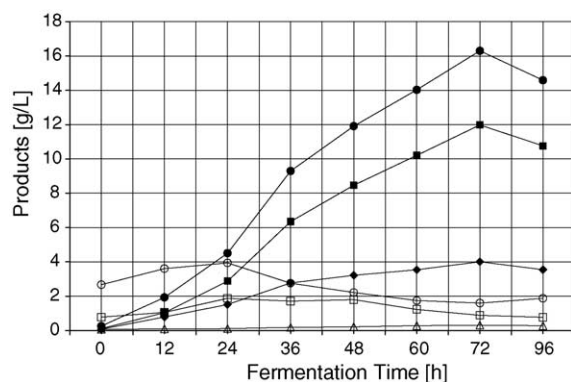


Fig. 3. Effect of retrograded starch sediments on acetone butanol ethanol fermentation by *C. beijerinckii* BA101 in semidefined P2 medium containing corn and soluble starch. Symbols: (Δ) ethanol; (□) butyric acid; (○) acetic acid; (◆) acetone; (■) butanol; (●) total solvent.

fluctuations in ABE concentration (Fig. 2a). The maximum total acid concentration measured in the fermentation effluent was 3.3 g L^{-1} . However, when the feed was maintained at room temperature (19°C), lower concentrations of ABE were observed in the reactor, although the fermentation did not stop until it was intentionally terminated at 504 h. These results demonstrate that gelatinized soluble starch may not be completely free from the affects of retrogradation. Production of acids during soluble starch fermentation is shown in Fig. 4a. In addition to investigation of fermentation parameters, we also investigated whether *C. beijerinckii* BA101 hydrolyzed starch to glucose faster than it utilized the latter for cell growth, maintenance, and ABE production. Cultures were able to hydrolyze starch efficiently and there was free glucose present in the fermentation medium throughout the fermentation process as can be seen in Fig. 4b. These results indicated that the extracellular amylases continued hydrolyzing soluble starch efficiently throughout the fermentation, unlike when corn starch was used.

3.3. Effect of storage on the susceptibility of gelatinized corn and soluble starch to enzymatic hydrolysis

Deterioration (retrogradation) of starch in feed, which was measured in terms of degree of hydrolysis during continuous cultivation of *C. beijerinckii* BA101 can be seen in Fig. 5. At 24 h of continuous cultivation

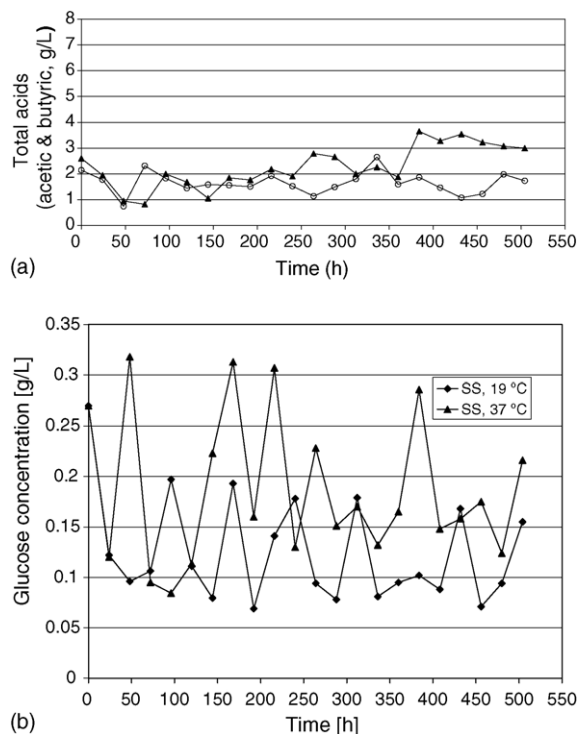


Fig. 4. (a) Total acid concentrations obtained during continuous cultivation of *C. beijerinckii* BA101 in semidefined P2 medium containing soluble starch. Symbols: (▲) soluble starch (19°C); (○) soluble starch (37°C). (b) Residual glucose concentrations obtained during continuous cultivation of *C. beijerinckii* BA101 in semidefined P2 medium containing soluble starch. Symbols: (◆) soluble starch (19°C); (▲) soluble starch (37°C).

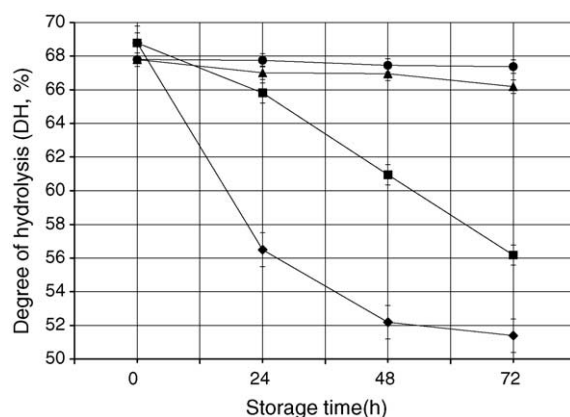


Fig. 5. Effect of storage of gelatinized corn starch and soluble starch on the susceptibility to hydrolysis by α -amylase enzyme. Symbols: (▲) soluble starch (19°C); (●) soluble starch (37°C); (◆) corn starch (19°C); (■) corn starch (37°C).

of *C. beijerinckii* BA101, corn starch feed which was stored at 37 °C, demonstrated a 3.0% decrease in degree of hydrolysis compared to a 12.3% decrease in degree of hydrolysis when the feed storage temperature was 19 °C. At 72 h of storage, the degree of hydrolysis of starch feed stored at 37 °C decreased by an additional 9.6% (total decrease in degree of hydrolysis, 12.6%), while corn starch feed stored at 19 °C demonstrated an additional 5.5% (total decrease in the degree of hydrolysis, 17.8%) decrease in degree of hydrolysis. On the other hand, soluble starch feed whose storage temperature was 19 °C demonstrated only 1.6% decrease in degree of hydrolysis after 72 h, while soluble starch feed stored at 37 °C showed no apparent change in the degree of hydrolysis.

4. Discussion

The results obtained during the batch fermentation of corn starch in P2 medium demonstrated that hyperamylolytic *C. beijerinckii* BA101 was capable of producing solvents from starch which is comparable to when glucose is used during batch fermentation (Table 1). A plot of starch utilization by *C. beijerinckii* BA101 during batch fermentation demonstrated that *C. beijerinckii* BA101 does utilize starch efficiently during the batch fermentation process (Fig. 1).

Continuous cultivation of *C. beijerinckii* BA101 in P2 medium containing corn starch whose feed supply was maintained at either 19 or 37 °C showed differences in ABE production, duration of fermentation and residual glucose concentration in the reactor (Fig. 2a and b). Starch utilization by *C. beijerinckii* BA101 for ABE production depends on the ability of the microorganism to secrete relevant and adequate amylolytic enzymes necessary for complete breakdown of starch to glucose. Figs. 2a and b shows that the fermentation was prematurely terminated during continuous cultivation of *C. beijerinckii* BA101 in P2 medium containing corn starch whose feed supplies were maintained at 19 and 37 °C due to inability of the amylolytic enzymes from *C. beijerinckii* BA101 to hydrolyze retrograded starch to glucose in the bioreactor. This resulted in higher levels of acid being produced in the bioreactor (5.00–7.97 g L⁻¹). It should be noted that in ABE fermentations, acids are metabolic intermediates and are generally produced before they are reassimilated for

acetone and butanol production. However, reassimilation of acids for conversion to ABE by *C. beijerinckii* BA101 seemed not to occur when the carbon source (glucose) in the fermentation medium is in a limited supply. The presence of a limited amount of glucose in the bioreactor is known to result in predominantly acid and low ABE production levels (Qureshi and Maddox, 1987; Campos et al., 2002).

Interestingly, when soluble starch was used as carbon source in P2 medium during continuous cultivation of *C. beijerinckii* BA101, the bioreactor was operated at the same dilution rate for 21 days without any sign of acid drift or cell morphological changes. The bioreactor whose feed supply was maintained at 37 °C produced higher total solvents, lower acid and higher residual glucose concentrations than the bioreactor whose feed supply was maintained at 19 °C (Fig. 2a and Fig. 4a and b). The fermentation in the both cases did not self-terminate. Most interestingly, the culture was able to hydrolyze soluble starch to glucose (Fig. 4b) faster than it utilized the latter for cell growth, maintenance, and ABE production. This may explain why *C. beijerinckii* BA101 maintained relatively low acid concentration in the bioreactor throughout the fermentation period (Fig. 4a) and the fermentation did not stop until it was intentionally terminated.

Retrogradation renders the starch less available to amylolysis (Kim et al., 1997). This study was carried out in order to determine why gelatinized corn starch does not support the continuous cultivation of *C. beijerinckii* BA101 after 312 h of operation. The effect of storage on the susceptibility of gelatinized corn starch indicated that the degree of hydrolysis of gelatinized corn starch decreased with time (Fig. 5). It should be noted that the physical and chemical properties of gelatinized starch solutions change during storage time due to retrogradation. Retrogradation of gelatinized corn starch was greater when the feed was stored at 19 °C than when it was stored at 37 °C as shown in Fig. 2b, Fig. 4b and Fig. 5. These results explain why P2 medium containing corn starch stored at 37 °C resulted in a fermentation which lasted up to 432 h (Fig. 2a and b). The relatively low ABE production following the continuous cultivation of *C. beijerinckii* BA101 in P2 medium containing corn starch was due to the retrogradation of the corn starch making it less susceptible to enzymatic hydrolysis. This resulted in the presence of a limited amount of glucose in the bioreactor, which was

insufficient to maintain the continuous cultivation of *C. beijerinckii* BA101. The low concentration of glucose (Fig. 2b) in the bioreactor resulted in the process being primarily acidogenic instead of solventogenic. The accumulated sediment was retrograded starch, which is resistant to enzymatic hydrolysis and although not toxic to the microorganism, does result in unfavorable growth conditions for *C. beijerinckii* BA101 in the reactor. However, an analysis of the degree of hydrolysis of soluble starch in P2 medium maintained at 37 °C showed no apparent change in the degree of hydrolysis (Fig. 5). Continuous cultivation of *C. beijerinckii* BA101 in soluble starch maintained at 37 °C resulted in sufficient amounts of glucose in the bioreactor thereby keeping the process solventogenic as demonstrated by the lower total acids maintained in the bioreactor throughout the fermentation.

A practical way for maintaining the long-term stable continuous cultivation of *C. beijerinckii* BA101 has been presented in this work. It was found that during continuous cultivation of *C. beijerinckii* BA101, the culture utilized soluble starch (which is less susceptible to retrogradation) better than native corn starch. It is concluded that retrogradation, which may lower the susceptibility of gelatinized starch to enzymatic hydrolysis, plays a role in the premature termination of the continuous fermentation process (when native corn starch was used) since the maintenance of culture metabolism depends on the availability of simple sugars (hydrolysis products) being available in the bioreactor. Continuous fermentation using starch as carbon source can be sustained for a longer period of time if the gelatinized starch is stabilized by eliminating or drastically reducing retrogradation. Furthermore, retrogradation of corn starch is slowed when the feed supply is maintained at 37 °C

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